C2

number of atoms and/or molecules in a virion constrained nanoparticle is determined by the size of the nanoparticle and the size of the virion inner cavity.

REMARKS

Reexamination and reconsideration in light of the foregoing amendments and the following remarks is respectfully requested.

Claims 21-38 are pending in this application. Claims 22 and 25 have been amended to correct obvious errors. A marked-up version of the changes to these claims appears in APPENDIX A attached hereto. No new matter has been added to the claims. Claim 22 has been amended to delete the term "plant". The inclusion of the term would be inconsistent with the base claim. As for claim 25, this claim has been amended to change the first word in the claim from "A" to "The".

Applicants note the Examiner's consideration of the references cited in the Information Disclosure Statement filed December 12, 2000.

Claim 21-28 stand rejected under 35 U.S.C. §112, first paragraph, as being based upon a non-enabling disclosure. According to the Examiner, the disclosure "does not reasonably provide enablement for composition containing [non-plant] virion-constrained nanoparticles."

The test of enablement is whether one skilled in the art could make or use the claimed invention from the disclosures in the specification coupled with information known in the art <u>without undue experimentation</u>. *United States V. Telectronics, Inc.*, 857 F.2d 778, 785, 8 USPQ2d 1217, 1223 (Fed. Cir. 1988), *cert. denied*, 490 U.S. 1046 (1989); *In re Stephens*, 529 F.2d 1343, 1345, 188 USPQ 659, 661 (CCPA 1976). The determination of enablement is a

question of law based on underlying factual findings. *In re Vaeck*, 947 F.2d 488, 495, 20 USPQ2d 1438, 1444 (Fed. Cir. 1991); *Atlas Powder Co. v. E.I. Du Pont De Nemours & Co.*, 750 F.2d 1569, 1573, 224 USPQ 409, 411 (Fed. Cir. 1984). In determining whether a disclosure would require undue experimentation to make the claimed subject matter, the Examiner must consider the quantity of experimentation necessary, the amount of direction or guidance presented, the presence or absence of working examples, the nature of the invention, the state of the prior art, the relative skill of those in the art, the predictability or unpredictability of the art, and the breadth of the claims. *In re Wands*, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404, (Fed. Cir. 1988), citing with approval *Ex parte Forman*, 230 USPQ 526, 547 (Bd. Pat. App. & Int. 1986). The burden is on the Examiner to establish a reasonable basis to question the adequacy of Applicants' disclosure. *In re Marzocchi*, 439 F.2d 220, 223-224, 169 USPQ 367, 370 (CCPA 1971).

The Examiner has noted the factors in *Wands* and finds that undue experimentation would be required because the "disclosure only provides a single working embodiment while the prior art clearly provides a number of rational scientific caveats (e.g., nanoparticle instability due to particle aggregation; inhomogeneous particle size distributions; instability of host matrices; inability to extract the material from the host matrix; and inability to control gating mechanisms) that would preclude the skilled artisan from practicing the claimed invention." It is Applicants' position that the Examiner has not established a *prima facie* case for a finding of lack of enablement.

The method of the claimed invention is quite simple. The first step is to provide an isolated and substantially pure coat protein of the non-plant virion. The second step is to

incubate the protein in a solution comprising the material to be entrapped in the coat protein. The third step is to provide conditions in which the nanoparticles are surrounded by the protein and then isolating the virion-constrained nanoparticles. Two examples are given in the application detailing this process with a CCMV virion.

The Examiner asserts that "[i]t is art-recognized that the mechanisms of viral assembly are complex and poorly understood." As evidence for this conclusion, the Examiner relies on an article by Dong et al. According to the Examiner, Dong et al. disclose that the "nature of protein-protein interactions during retrovirus assembly is not well understood, and molecular genetic analyses of functional regions within the gag and env gene products are only beginning to provide information in this regard." This is an observation only. The Examiner has not explained (i) how the lack of understanding of the nature of protein-protein interactions during retrovirus assembly relate to encapsulation using a non-plant virion and why such lack of understanding would require undue experimentation by a person having ordinary skill in the art and (ii) how the gag and env gene products referred to would render non-enabling the encapsulation of an organic or inorganic material by a non-plant virion. The Dong et al. reference refers to mechanisms involving viral and cellular proteins, but the Examiner has not explained by cogent scientific reasoning how the lack of understanding of these mechanisms would have led to undue experimentation to practice the present invention.

Copies of the Declarations Under 37 CFR § 1.132 of Adam Zlotnick, Ph.D., Thomas J. Smith, Ph.D., and John E. Johnson, Ph.D., which were filed in the parent of this application, Application No. 08/775,366, are attached in APPENDIX B. Each of the declarants are skilled in the art (Zlotnick, p. 2, ¶ 5; Smith, p. 3, ¶ 5; and Johnson, p. 3, ¶ 5) and declare that

> the Examiner also refers to a publication by Dong et al. …, as suggesting that the prior art teaches that the mechanisms of viral assembly are complex and poorly understood. This publication was made in 1993, well prior to the filing of this patent application. This patent application presents a clear description of how controlled gating may be used. It is clear that Dong had no concept of the gating mechanism of this invention and should not be used as evidence to raise questions about the gating mechanism of this invention because Dong et al. is not concerned with the same processes. Once again, the claims of the inventors are not addressing the multitude of viral assembly mechanisms. They claim only that given a stable empty viral protein cage to which there is access to the virion's interior that it can be used as a constrained reaction vessel for selective material entrapment. [Zlotnick Declaration, p. 9, ¶ 13; Smith Declaration, p. 9, ¶ 13; and Johnson Declaration, p. 9, ¶ 13.]

Dong et al. does not represent the state of the art from which to conclude that one skilled in the art would find Applicants' disclosure non-enabling. Dong et al. does not represent the state of the art as of the effective date of filing of the parent patent application, namely, January 1997. The reference was published in 1993, four years before the filing of Applicants' earliest filing date.

The Examiner made a finding that the field of molecular nanotechnology is in its infancy, and presumably is unpredictable, such that a person having ordinary skill in the art would have to engage in undue experimentation to encapsulate organic and inorganic materials using a non-plant virion. For this finding, the Examiner relies on an article published by Kaehler in 1994. According to the Examiner, Kaehler "reviews the state-of-the-art and concludes that while there are many potential applications for nanotechnology, these applications have yet to be realized." The reference only establishes the state of the art in 1994, and not the state of the art or level of skill in the art as of the effective filing date of the present application. Moreover, a field being in its infancy is not, in and of itself, sufficient to establish that a person having ordinary skill in the art would been required to engage in undue experimentation to practice the processes disclosed

in the application. The Examiner refers to the limited number of applications in nanotechnology. The applications in nanotechnology are not the issue here. The issue is whether the non-plant virions will encapsulate organic or inorganic material. The state of the art which has been presented by the Examiner does not establish a state of the art that virions are unpredictable and that non-plant virions would be expected to perform substantially differently from non-plant virions. The Examiner has not presented any evidence to establish this fact. The field of molecular nanotechnology is a growing technology as evidenced by the published articles attached as APPENDIX C. The articles describe enablement and the uses being made of nanotechnology.

The Examiner further relies on an article by Douglas et al. published in 1987. This article discusses the state of the art in 1987, and does not discuss the state of the art in 1997.

Dong et al., Kaehler and Douglas (1987) taken together fail to establish the state of the art or level of skill in the art as of the effective filing date of the present patent application in 1997.

The Examiner relies on an article by Douglas published in 1996 as providing "an overview of the biomimetic synthesis of nanoscale particles in organized protein cages." The Examiner asserts that the reference discloses that a number of limitations have precluded advancement of the technology including "instability to particle aggregation, inhomogeneous particle size distributions, insolubility of hosts matrices, and an inability to extract the material from the host matrix." The claims are directed to a virion-constrained nanoparticle comprising a non-plant virion coat protein shell surrounding a nanoparticle and a process for producing the same. The Examiner has not explained how and why particle aggregation, inhomogeneous particle size distributions, insolubility of host matrices and the inability to extract the material

from the host matrix would render the claimed subject matter non-enabling and why a person having ordinary skill in the art would be required to engage in undue experimentation to practice the claimed invention. It is totally unclear why inability to extract the material from the host matrix is relevant to the claimed subject matter since the claims are not directed to extracting the encapsulated material. Also, the rejection lacks any explanation based on scientific reasoning as to why and how particle aggregation, particle size and insolubility of host matrices are relevant to the claimed method of making an protein encapsulated nanoparticle or to the claimed virion constrained nanoparticle, and why such factors would lead to undue experimentation.

As set forth *supra*, Declarants Zlotnick, Smith and Johnson are persons skilled in the art. Their declarations are evidence that the limitations referred to by the Examiner would not require undue experimentation to practice the claimed invention. With respect to the 1996 Douglas publication, each of the declarants states that

> the Examiner, ... refers ... to the publication by Douglas ... (1996), as indicating that a number of limitations have precluded the advancement of synthesis of nanoscale particles into organized protein cages. This is the inventor Trevor Douglas's own publication and simply indicates on page 92 some difficulties to overcome problems with instability to particle aggregation and the like. However, this publication was made before the inventors completed their invention. There is evidence in the record from Dr. Douglas which clearly refutes these earlier writings. The Examiner therefore is relying on the publication by the inventor made before the invention was made. This publication is overcome by the inventors' representation in this patent application and Dr. Douglas's Declaration of record. [Zlotnick, p. 8, ¶ 11; Smith, p. 8, ¶ 11; and Johnson, p. 8, ¶ 11.]

The Douglas Declaration, which was of record in parent Application No. 08/775,336, is attached hereto as APPENDIX D. In his declaration, Douglas states that "it is my scientific opinion that the subject invention represents a general method for encapsulating a wide variety of organic and

inorganic material within the interior cavities of both RNA-containing and RNA-depleted virions without undue experimentation" (Douglas Declaration, p. 3, ¶ 5). The record of this application does not present any evidence to cast doubt that Douglas' statement or aforementioned statement from Declarants Zlotnick, Smith and Johnson are not true.

The Examiner also relies on Howk et al. published in 1996 as teaching the state of the art and level of skill in the art. Declarants Zlotnick, Smith and Johnson, persons skilled in the art, declare in each of their declarations the following:

> The Examiner ... refers to a publication by Howk et al. ... as disclosing several concerns regarding gating as a control element for nanoparticle loading. While the Examiner points to concerns raised in this article, what is actually concluded in the Abstract is that gating has a critical influence on the ease of formation and stability of host guest complexes and that hosts equipped with gates can form very stable complexes with a variety of guests under readily achievable conditions. Therefore, this publication, relied on by the Examiner as suggesting concerns, actually shows that when used correctly, gating can be used as a control element under readily achievable conditions. Therefore, this article refutes the Examiner's suggestions. [Zlotnick Declaration, p. 8, ¶ 12; Smith Declaration, p. 8, ¶ 12; and Johnson Declaration, p. 8, ¶ 12.]

There is no evidence of record to cast doubt on the truthfulness of this statement. The Examiner has not explained how gating would have led a person having ordinary skill in the art to engage in undue experimentation to practice the claimed invention or why a lack of disclosure to gating would render the specification non-enabling. The Examiner made a finding from Houk et al. that "some host molecules have small portals that preclude guest or solvent passage through the molecule into the interior of the particle, other hosts have large portals that are not readily influence and fail to form any stable complex with the guest molecule, and finally, some host molecules have small portals that admit guest molecules only under specific solvent conditions." The Examiner has not explained how and why this finding would cause undue experimentation

with respect to the non-plant virion coat protein shells of the invention. The finding is merely a general observation, but, in and of itself, does not establish undue experimentation would be required.

The specification defines the types of "virions" which can be employed in practicing the invention as including prokaryotic, protozoan, algal, fungal and eukaryotic viruses. The disclosure sets forth examples of each of these types of "virions". No evidence or cogent scientific reasoning has been presented by the Examiner to establish that undue experimentation would be required to practice the invention with respect to non-plant virions. Each of the Declarants Zlotnick, Smith and Johnson refer to the Declaration of Michael J. Young, which is of record in parent Application No. 08/775,366. A copy of the Young Declaration is attached as APPENDIX E. Declarants Zlotnick, Smith and Johnson state:

> The Declaration of Dr. Young provides the results of additional experiments which indicates that structures of more than 30 viruses have been determined to atomic resolution and that these structures reveal that the coat protein subunits of all virions are assembled and stabilized by non-covalent bond interactions such as H bonding ionic interactions and hydrophobic interactions. Further, Dr. Young's Declaration states that the vast majority of icosahedral viruses have a coat protein subunit that utilizes a 8-stranded anti-parallel, _-barreled fold, commonly termed the "_barrel jelly roll fold", which protein fold is dominant across all taxmomic classes of virus regardless of host. Dr. Young then lists various viruses CCMV, the human viruses Norwalk virus, Polio virus, Rhino virus, Parvo virus and Flockhouse virus which have this protein fold as the predominant structural feature. Dr. Young then presents evidence as having synthesized a paratungstate polymer using the virions protein cage of an animal Noralk [sic, Norwalk] virus (NWV) having icosheidral geometry and a constrained reaction vessel. Dr. Young also presents evidence of encapsulation of paratungstate within CCMV, and encapsulation of polyanetholesulfonic acid within CCMV, encapsulation of iron oxides within CCMV. Dr. Young then concludes that every system studied by the inventors has meet with success with only minor modifications being required in some cases. [Zlotnick, p. 6, ¶ 10; Smith, p. 7, ¶ 10; and Johnson, p. 7, ¶ 10.]

There is no evidence of record to cast doubt on the truthfulness of the statements made by the declarants. According to Declarant Young, the predominant structural feature defining the protein shell referred to above by Declarants Zlotnick, Smith and Johnson, "may ultimately account for our observations to date revealing the broad capacity of viral capsids to act as constrained reaction vessels for mineralization and/or entrapment of inorganic, organic and metallo-organic substances" (Young Declaration, p. 3, ¶ 4). Since the structure set forth above appears to transcend plant and non-plant virion coat protein shells, persons having ordinary skill in the art would have expected an animal protein to exhibit the same encapsulation property as the CCMV virus. Applicants have presented evidence by persons skilled in the art, namely, the Declarations by Zlotnick, Smith and Johnson, that establish that following the teachings of the present disclosure, a person having ordinary skill in the art could practice the invention with non-plant virions without undue experimentation. There is no evidence of record or reasoning presented in the rejection of record that that would cast doubt on the truthfulness of statements made by Declarants Young, Zlotnick, Smith and Johnson.

The Examiner asserts that the specification "does not describe the preparation of virion-constrained nanoparticles from any other virus, excluding CCMV." It appears to be the Examiner's position that the specification must include a working example of a non-plant virion. Such a working example is not required to establish enablement. *See In re Strahilevitz,* 668 f.2d 1229, 212 USPQ 561 (CCPA 1982) (working examples are desirable, but not necessarily required to satisfy enablement requirement). An inventor need not explain every detail of the invention. Patents are not production documents, and nothing in the patent law requires that the inventors disclose examples for each and every virion which is disclosed in the specification.

See DeGeorge v. Vernier, 768 f.2d 1318, 226 USPQ 758 (Fed. Cir. 1985); *Christianson v. Colt Industries Operating Corp.*, 822 f.2d 1544, 3 USPQ 2d 1241 (Fed Cir 1987).

The Examiner asserts that the specification "fails to provide adequate guidance concerning a number of these considerations as they pertain to the claimed invention." This is a conclusionary statement which has not been corroborated by any evidence. The Examiner asserts that the "disclosure only provides a single working embodiment while the prior art clearly provides a number of rational scientific caveats (e.g., nanoparticle instability due to particle aggregation, inhomogeneous particle size distributions; insolubility of host matrices; inability to extract the material from the host matrix; and inability to control gating mechanisms) that would preclude the skilled artisan from practicing the claimed invention." The Examiner further asserts that "Applicants fail to provide any guidance pertaining to any of these critical parameters" and that "[a]ccordingly, when the aforementioned factors are considered *in toto,* it would clearly require undue experimentation from the skilled artisan to practice the claimed invention."

These are all conclusionary statements. No evidence or cogent scientific reasoning based on teachings of the prior art or any evidence has been presented to explain why and how the identified "scientific caveats" would have precluded the skilled artisan from practicing the claimed invention because undue experimentation would be required. All that the Examiner has asserted is that these caveats exist without giving reasons why they would have led to undue experimentation. As noted *supra*, it is not the type of virion that controls encapsulation, but its structure as evidenced by the Declarations of Young at p. 3, ¶ 3 and the Declarations of Zlotnick, Smith and Johnson at p. 6, ¶ 8. Therefore, the Examiner has not established that the disclosure

lacks guidance with respect to encapsulating organic or inorganic materials with non-plant virions.

The Examiner has tried to establish that the nature of the invention is such that there is a speculation by a person skilled in the art as to the activity of virions. As discussed *supra*, the Examiner has not established that such activity is related to virion encapsulation of organic and inorganic materials.

Also as discussed, *supra*, the absence of a working embodiment or example is considered in determining non-enablement, but the absence of a working embodiment is not necessary to establish enablement. Since the evidence of record establishes that the structure of the virion that governs encapsulation, and not its type, i.e., whether the virion is a plant virion or non-plant virion, a person having ordinary skill in the art, as evidenced by the Declarations of Zlotnick, Smith and Johnson, would have found the plant virion working example sufficient to lead one to expect that the invention can be practiced using a non-plant virion in place of the plant virion without undue experimentation.

The Examiner has not presented any evidence or scientific reasoning based on such evidence that would establish that a significant quantity of experimentation would be necessary to cause the virions to open then encapsulate and close around the organic or inorganic particles. Moreover, the record of this application does not explain why the "scientific caveats" would require or lead to an excessive quantity of experimentation. To merely cite caveats without further explanation is inadequate to establish that an excessive quantity of experimentation would be required to practice the invention with non-plant virions.

For reasons already discussed, *supra*, the Examiner has not established that the state of the art or level of skill in the art at the time of the effective filing date of the present application. The Examiner has not established the relative skill of those in the art at the time of the effective date of the application. The Declarations of Zlotnick, Smith and Johnson, who are persons skilled in the art, establish the skill in the art. According to these persons, a person having ordinary skill in the art would have expected that the invention could be practiced using non-plant virions without undue experimentation.

As for the breadth of the claims, the claims are directed to non-plant virions and for reasons discussed, *supra*, the disclosure would be enabling to make and use the non-plant virions.

For all of the foregoing reasons, it is Applicants' position that the Examiner has not established a *prima facie* case that the disclosure of the present invention is non-enabling with respect to the claimed subject matter. The determination of lack of enablement is fact dependent. On the present record, the Examiner has not presented sufficient factual findings to establish that the disclosure does not satisfy the enablement requirement of 35 U.S.C. §112, first paragraph. Accordingly, it is respectfully requested that the rejection be reconsidered and withdrawn.

Accordingly, favorable reconsideration of claims 21-38 is requested in light of the preceding amendments and the remarks. Allowance of the claims is courteously solicited.

To the extent necessary, a petition for an extension of time under 37 C.F.R. 1.136 is hereby made. Please charge any shortage in fees due in connection with the filing of this paper, including

extension of time fees, to Deposit Account 500417 and please credit any excess fees to such deposit account.

Respectfully submitted,

McDERMOTT, WILL & EMERY

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APPENDIX A

VERSION WITH MARKINGS TO SHOW CHANGES MADE

Please amend claims 22 and 25 as follows:

22. (Amended) The [plant] virion-constrained nanoparticle according to claim 21, wherein said nanoparticle of non-viral origin comprises an organic material.

25. (Amended) The [A] virion constrained nanoparticle according to claim 21, wherein said virion constrained nanoparticle comprises particles having dimensions substantially in the nanometer range and which comprise a collection of atoms and/or molecules ranging in number from 1 to the number that can fit inside the volume of the selected virion whereby the maximum number of atoms and/or molecules in a virion constrained nanoparticle is determined by the size of the nanoparticle and the size of the virion inner cavity.

APPENDIX B

Declarations Under 37 CFR § 1.132 of Adam Zlotnick, Ph.D.,
Thomas J. Smith, Ph.D., and John E. Johnson, Ph.D.

Docket No.: 50198-079 (228-053) **PATENT**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	:	
Trevor DOUGLAS et al.	:	Group Art Unit: 1648
Serial No. 08/775,366	:	Examiner: J. Parkin
Filed: January 3, 1997	:	

For: NANOSCALE PARTICLES SYNTHESIZED WITHIN AN ASSEMBLED VIRION

RULE 132 DECLARATION OF MARK J. YOUNG

Honorable Commissioner of
Patents and Trademarks
Washington, D. C. 20231

Sir:

I, Mark J. Young, Ph.D., do hereby declare and affirm that:

1. I am a co-inventor in the above-captioned patent application.

2. I received a Ph.D. degree in Plant Virology in 1987 from the University of California, Davis, and am currently an Associate Professor in the Department of Plant Sciences at Montana State University. A copy of my Curriculum Vita is attached herewith as an Appendix.

3. I wish to make a few observations concerning the biochemical processes occurring in the present invention and to relate the results of additional experiments that have recently been performed by myself and/or Trevor Douglas, a co-inventor of this application. These results provide still further support for our belief and position that the invention described in the

present patent application represents a major contribution to the field of nanotechnology that is "pioneering" in nature.

4. Background. By definition, all viruses form a protein shell (capsid) that serves to protect and transport viral nucleic acid. The protein shell inherently defines a shape and size constrained interior cavity for the viral nucleic acid. The architectures of all virus protein shells are thought to be geometric in shape and thus follow the rules of geometric symmetry. This is due to the requirement that all viruses follow the rule of 'genetic economy', i.e., the need to assemble the protein shells using multiple copies of one or a few coat protein subunits. This is true of all viruses regardless of their host (animal, bacteria, plant, insect, etc.) and whether or not the virion is enveloped in a lipid bilayer.

To date the structures of more than 30 viruses have been determined to atomic resolution. These include examples of both icosahedral and helical-based virion architectures. These structures reveal that the coat protein subunits of all virions are assembled and stabilized by non-covalent bond interactions (principally H-bonding, ionic interactions, and hydrophobic interactions). The vast majority of icosahedral viruses have a coat protein subunit that utilizes an eight-stranded, anti-parallel, beta-barrel fold (commonly termed the 'beta-barrel jelly roll' fold). This protein fold is dominant across all

taxonomic classes of virus regardless of host (animal, plant, insect, bacteria, and fungi). The plant virus cowpea chlorotic mottle virus (CCMV), the human viruses Norwalk virus (NWV), poliovirus, rhinovirus, parvovirus, and the insect Flockhouse virus (FHV), to cite just a few examples, all have the beta-barrel jelly roll fold as the predominant structural feature defining their protein shells. The ubiquitous occurrence of this motif among the known structures of viral capsids suggests that it is essential to the structural stability and/or assembly of all viral coat proteins. Without wishing to be bound by any particular theory, this apparently universal structural feature may ultimately account for our observations to date revealing the broad capacity of viral capsids to act as constrained reaction vessels for mineralization and/or entrapment of inorganic, organic and metallo-organic substances.

A detailed analysis of the known atomic resolution structure of viruses, coupled with recent studies on the dynamic nature of many virions, indicates that virions possess pores (holes) in their protein shell that allow access from the exterior to the interior of the virions. Many (most) of these pores are independent of the gating mechanism that we have explored in our original CCMV studies. In CCMV, an icosahedral structure, even in its closed (unswollen) conformation, the crystal structure reveals pores at the 3-fold axis approximately 10Å in diameter.

In addition, NWV has 10-13Å holes in each of its twelve pentameric vertices, which allow access from the outside to the virion interior. We suspect that our successful synthesis of a paratungstate polymer within the NWV interior (described below) is driven by passage of tungstate ions through these holes into the virion interior. Similarly, the helical tobacco mosaic virus (TMV) has a 4Å channel extending the entire 300 nm length of the virus particle. It is likely that this channel allows iron ions to pass during the mineralization of iron oxide in this central core channel. Many other viruses, independent of the host source, have pores in their protein shell; for example, poliovirus, human rhinovirus, and the insect Flockhouse virus. <u>The presence of these pores in many virions indicates that the present use of virion protein "cages" as constrained reaction vessels is not limited to those virions capable of undergoing "reversible gating"</u>.

5. As described below, we have synthesized a paratungstate polymer using the virion protein cage of an animal Norwalk virus (NWV) having icosahedral geometry as a constrained reaction vessel, in addition to our earlier work using CCMV and the helical tobacco mosaic virus (TMV). This demonstrates that the present concept of using virions as constrained reaction vessels for material synthesis and molecular encapsulation is not limited to plant viruses, but can be extended to animal viruses, and

logically to any virus having a protein shell that defines a size and shape constrained interior cavity. Further evidence to support this claim is demonstrated by the fact that we have also successfully synthesized an iron oxide mineral in the helical TMV. This clearly demonstrates that the present concept of using virions as constrained reaction vessels encompasses both icosahedral and helical viruses, which span the spectrum of virus taxa and is completely independent of the taxonomic classification of the host that the virus is originally derived from.

6. Encapsulation of paratungstate within an empty Norwalk virion: We have successfully entrapped a paratungstate mineral within the central cavity of the animal Norwalk virus (NWV). Assembled NWV virions devoid of nucleic acid were purified from insect cell cultures that expressed the major NWV coat protein using baculovirus-directed heterologous protein expression technology. The empty virus particles isolated from the insect cell culture system appear morphologically indistinguishable from infectious NWV particles isolated from infected humans. The purified empty NWV particles were incubated in the presence of 25mM Na_2WO_4, 75 mM NH_4Cl for 24 hours at 22^0C. After incubation, the sample was extensively washed with 0.1M NaAc to remove all WO_4^{2-} present in the bulk medium. Samples were subsequently examined by transmission electron microscopy. Examination of

unstained samples revealed the presence of spherical electron dense cores of a size and shape corresponding to the predicted size of the interior cavity of the empty NWV particles. Control samples of NWV incubated in the absence of Na_2WO_4 did not have such spherical electron dense cores. Likewise, incubations in the absence of NWV particles failed to produce the spherical electron dense cores. These results clearly demonstrate that animal viruses, like plant viruses, can be utilized as constrained reaction vessels. This result demonstrates that the ability to use virion protein cages as constrained reaction vessels is not limited to those viruses having a plant host.

7. Encapsulation of Decavanadate within CCMV: Empty CCMV virions (50µg) were incubated with 160 mM $NaVO_3$ at pH 6.5 for 1 hour. An equal volume of 100 mM NH_4Cl, pH 3.0 was added resulting in the formation of a bright yellow-orange solution containing the $V_{10}O_{28}^{6-}$ anion. After 24 hours at 6°C the reaction mixture was concentrated and washed extensively with NH_4Cl (pH 4.0), using ultra-centrifugation. Examination by TEM revealed the formation of monodisperse nanoparticles with essentially the same size distribution as observed for polytungstate. Negative staining shows an intact protein coat. Control reactions in the absence of the absence of virion yielded yellow-orange crystals after slow evaporation of water at 6°C. These crystals exhibited X-ray powder diffraction patterns consistent with a mixture of

$Na_6V_{10}O_{28} \cdot 18H_2O$ and $(NH_4)_6V_{10}O_{28} \cdot 18H_2O$.

8. Encapsulation of Polyanetholesulfonic acid within CCMV: Empty virions (100 µg) were incubated in a 20 mg/mL polyanetholesulfonic acid (average molecular 9,000 - 11,000), 50 mM Tris-HCl pH 7.5 solution at 25°C. After incubation, the pH of the solution was decreased to pH 4.5 with 100 mM sodium acetate buffer and washed extensively with acetate buffer in a Centricon 100. The sample was subsequently loaded onto, and centrifuged through, a 10% - 40% sucrose gradient. Gradient fractions were collected and monitored by absorption at 280 nm and 315 nm. Gradient fractions were washed and concentrated using Centricon 100 ultrafilters. Samples were visualized by TEM. A strong, unique, absorption characteristic of polyanetholesulfonic acid (315 nm) was only present in virion containing fractions. The controls of initially incubating an equivalent amount of empty virions at pH 4.5 instead of pH 7.5 did not show absorption characteristic of polyanetholesulfonic acid.

8. Encapsulation of Iron oxides within CCMV: Empty virions (25 µg) in 10 mM MES buffer (pH 6.4, 25°C) were treated with aliquots of Fe^{2+} (25mM) and allowed to air oxidize (for 1 hr) between additions. This resulted in the formation of a homogeneous solution having a pale orange color, due to the presence of iron oxide nanoparticles within the virion. Control experiments, in the absence of the virion, resulted in the

formation of an orange precipitate. A portion of the liquid sample was dried down onto a sample holder and imaged by TEM at 80keV. Electron dense particles commensurate in size with the CCMV dimensions were observed. Negative staining of this sample revealed intact virions, which indicate that the interior cavity of the CCMV was mineralized.

9. <u>Encapsulation of a molecular dye species, 5,5′-dibromo-o-cresolsulfonphthalein (bromcresol purple) within CCMV:</u> Empty virions (100 µg) were incubated in a 2.0 mg/mL solution of the indicator dye bromcresol purple in 50 mM Tris-HCl pH 7.5 solution at 25°C. After incubation, the pH of the solution was decreased to pH 4.5 with 100 mM sodium acetate buffer and washed extensively with acetate buffer in a Centricon 100. The sample was subsequently loaded onto, and centrifuged through, a 10% - 40% sucrose gradient. Gradient fractions were collected and monitored by absorption at 280 nm and 412 nm. Gradient fractions were washed and concentrated using Centricon 100 ultrafilters. Samples were visualized by TEM. A strong, unique, absorption characteristic of bromcresol purple (412 nm) was only present in virion containing fractions. The controls of initially incubating an equivalent amount of empty virions at pH 4.5 instead of pH 7.5 did not show an absorption characteristic of bromcresol purple. This indicates that we have successfully entrapped an isolated molecular species within the virion (there

may be more than one dye molecule per virion but the volume of the virion interior is very large compared to the volume occupied by each dye molecule implying isolated non-interacting species).

9. Conclusion. We have shown that virions, devoid of their nucleic acid, can be used as containers for entrapment of nanoparticles. We have demonstrated this methodology using plant and animal viruses. In addition, we have demonstrated the method in both icosahedral (spherical) and helical (rod-shaped) virions. We have also demonstrated that we can entrap materials formed by aggregation of cationic species (iron oxides), polyanionic species (paratungstate, decavanadate, polyanetholesulfonic acid, polydextran sulfate), as well as isolated molecules (bromcresol purple). These syntheses and entrapments have been demonstrated within a single virion species (CCMV) as well as in different virion (CCMV, TMV, NWV) structures.

Moreover, since our original work demonstrating the encapsulation of paratungstate in CCMV, every system we have studied has met with success, with only minor modifications being required in some cases. Therefore, it is my view that the methods described in the above-referenced patent application are applicable generally to virions independent of their natural hosts or geometric structures, as well as the chemical nature of the encapsulated material.

Serial No. 08/775,366

The undersigned hereby declares that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 6/18/98

Mark J. Young, Ph.D.

APPENDIX

CURRICULUM VITA OF MARK J. YOUNG

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EDUCATION

Institution and location	Degree	Year conferred	Field of study
University of California, Davis, California, USA	Ph.D.	1987	Plant Virology
University of California, Berkeley, California, USA	B.A.	1980	Biochemistry
University of California, Berkeley, California, USA	B.Sc.	1980	Soils and Plant Nutrition

ACADEMIC APPOINTMENTS

Associate Professor 3/97 - present	Department of Plant Sciences, Montana State University.
Assistant Professor 9/94 - 3/97	Departments of Microbiology and Plant Pathology, Montana State University.
Assistant Professor 4/91 - 9/94	Department of Biological Sciences, Structural Biology Group, Purdue University.
Research Scientist 12/87 - 4/91	Division of Plant Industry, Canberra Australia.

B. PUBLICATIONS

Pertinent Publications:

1. Douglas, T., Young, M. J., "Host Guest Encapsulation of Materials by Assembled Virus Protein Cages", *Nature* (1998), 393, 153-155.
2. Fox, J., Albert, F., Speir, J., and M. J. Young. 1997. Characterization of a disassembly deficient mutant of cowpea chlorotic mottle virus. Virology 227:229-233.
3. Albert, F., Fox, J., and M. J. Young. 1997. Viron swelling is not required for cotranslational disassembly of cowpea chlortic mottle virus *in vitro*. J. Virology 71:4296-4299.

4. Fox, J., Zhao, X., and M. J. Young. 1996. Analysis of a salt stable mutant of cowpea chlorotic mottle virus. Virology 222:115-122.
5. Zhao, X., Fox, J., Olson, N., Baker, T., and M.J. Young. 1995. *In vitro* assembly of cowpea mottle virus from coat protein expressed in **E. coli** and *in vitro* transcribed viral cDNA. Virology 207:486-494.

Related Publications:
1. Fox, J., Johnson, J., and M.J. Young. 1994. RNA/protein interactions in icosahedral virus assembly. Semin. Virol. 5:51-60.
2. Fox, J., Wang, G., Olson,N., Speir, J., Johnson, J., Baker, T., and M. J. Young. 1997 Comparison of the native CCMV virion with *in vitro* assembled CCMV virions by cryo-electron microscopy and image reconstruction. Virology. (in press).
3. Filichkin, S., Brumfield, S., Filichkin, T., and M. J. Young. 1997. *In vitro* interations of the aphid endosymbiotic SymL chaperonin with barley yellow dwarf virus. J. Virology 71:569-577.
4. Filichkin, S., Lister, R., McGrath, P., and M. J. Young. 1994. *In vivo* expression and mutational analysis of the barley yellow dwarf virus readthrough gene. Virology. Virology 205:290-299.
5. Feyter, R. Young, M., Schroeder, K., Dennis, E., and W. Gerlach. 1996. A ribozyme gene and an antisense gene are equally effective in conferring resitance to tobacco mosaic virus on transgenic tobacco. Mol Gen Gent. 250:329-338.

C. COLLABORATIONS

T.S. Baker
T. Douglas
J.E. Johnson

D. GRADUATE AND POSTDOCTORAL TRAINING

Graduate Students:

James Fox	Xiaoxia Zhao	Lisa Kelly
Lynae Beiningen	Zilka Schumaker	Na Li

Postdoctoral scholars:

Fred Albert	Sergei Flichkin

E. PAST GRADUATE AND POSTDOCTORAL ADVISORS

George Bruening   R. J. Shepherd W.L. Gerlach

APPENDIX E

Rule 132 Declaration of Mark J. Young, Ph.D.

Docket No.: 228-053 PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	:	
Trevor DOUGLAS et al.	:	Group Art Unit: 1813
Serial No. 08/775,366	:	Examiner: J. Parkin
Filed: January 3, 1997	:	

For: NANOSCALE PARTICLES SYNTHESIZED WITHIN AN ASSEMBLED VIRION

RULE 132 DECLARATION OF TREVOR DOUGLAS

Honorable Commissioner of
Patents and Trademarks
Washington, D. C. 20231

Sir:

I, Trevor Douglas, do hereby declare and affirm that:

1. I am a co-inventor of the above-captioned patent application.

2. I received a Ph.D. degree in Inorganic Chemistry from Cornell University in 1991 and am an Assistant Professor of Chemistry at Temple University. A copy of my curriculum vita is attached herewith as an Appendix.

3. In addition to the experiments disclosed in the above-identified application, further experiments in support of the claimed subject matter have been conducted by myself and/or Mark Young, a co-inventor of this application.

4. The aforementioned experiments are detailed as follows:

A. Encapsulation of Dextran Sulfate within CCMV

Dextran sulfate, a heparin-like polysaccharide containing approximately 17% sulfur with up to three sulfate groups per glucose residue, was tested as an alternative organic polyanion for selective entrapment by the protein cage of cowpea chlorotic mottle virus (CCMV). Gradient-purified CCMV (50μg) was added to 4,000 Da dextran sulfate solution (1mM, total volume 100μL) at pH 7.0 for 4 hr at 4°C. After incubation, the pH was lowered to 5.0 and the sample was analyzed by separation on a 10-40% sucrose gradient centrifuged in a Beckman SW41 rotor for 4 hr at 38,000 rpm. The resulting (dextran sulfate filled) virions had a sedimentation velocity of 70S compared with 52S for empty CCMV cages. This approximate 20S increase in sedimentation velocity, together with transmission electron microscopy (TEM) analysis of gradient fractions, confirms that the CCMV protein cages were filled with dextran sulfate. Controls of CCMV protein cages incubated in the absence of dextran sulfate did not exhibit the 20S shift in S value or appear full by TEM analysis.

B. Tobacco Mosaic Virus

Tobacco Mosaic Virus (TMV) is a rod-shaped virus approximately 15 nm by 300 nm in size, with a central cavity running the entire length of the structure. The cavity is present in both RNA containing and RNA depleted TMV.

TMV (RNA containing) was treated with aliquots of Fe^{2+} at pH 6.2 and allowed to air oxidize (for up to 8 hr) between additions of Fe^{2+}. This resulted in the formation of a solution having a pale orange color, which was due to the presence of iron oxide nanoparticles. A portion of the liquid sample was dried down onto a sample holder and imaged by TEM at 80keV. Electron dense fibers commensurate in size with the TMV virion, were observed in addition to a small amount of non-specific precipitation, i.e., precipitate on the outside of the virus particle. Negative staining of this sample revealed intact virus shells, which indicates that the interior cavity of the TMV particle was mineralized.

5. The results of the aforementioned studies evidence that the present invention can be employed (1) to encapsulate organic molecules in CCMV, and (2) to encapsulate inorganic substances in other virions besides CCMV, in particular TMV. Based on the above results and those disclosed in the patent application, it is my scientific opinion that *the subject invention represents a general method for encapsulating a wide variety of organic and inorganic materials within the interior cavities of both RNA-containing and RNA-depleted virions without undue experimentation.*

6. With regard to the cited *Zhao et al.* article, this reference discloses a negative staining technique employing uranyl acetate, which is used to visualize their virions. This staining technique is much like that employed by us in Example 2 of the

patent specification. Although some of the uranyl acetate can be expected to penetrate into the cavity of the stained virions, this process is entirely nonselective. In other words, some of the uranyl acetate can be expected to precipitate on, or otherwise adhere to, the exterior of the virion coat protein. In addition, any uranyl acetate deposited in the interior of the virion cannot be retrieved, i.e., it is irreversibly deposited therein. Furthermore, the cited reference does not disclose or suggest a controlled gating, e.g., pH induced, encapsulation method as is described in the present application.

The undersigned hereby declares that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 12/2/97

Trevor Douglas

Trevor Douglas, Ph.D.

APPENDIX

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Education:

Post-Doctoral Fellow,	Bio-mineralization, University of Bath, United Kingdom. Advisor: Professor Stephen Mann.
Ph.D.,	Inorganic Chemistry (Minor: Analytical Chemistry) Cornell University, USA, May 1991. Advisor: Professor Klaus H. Theopold. Dissertation: "Synthesis and Studies of III-V Semiconductor Precursors; Preparation of Semiconductor Colloids"
B.A.,	Biochemistry/Chemistry, May 1986, University of California at San Diego, USA.

Positions Held:

Assistant Professor, Temple University	present
Assistant Professor, Montana State University - Billings, Montana	1995-1997
Lecturer, Ithaca College	1994-1995
Post-doctoral Fellow, Bath University	1992-1994
Research Assistant, Cornell University	1986-1991
Undergraduate Research, UCSD	1984-1986

Awards:

Outstanding Faculty Award (teaching) from The Associated Students of Montana State University (1996).

Joseph E. Meyer Award for Undergraduate Research, University of California at San Diego, 1986.

President's Undergraduate Fellowship, University of California at San Diego, 1985.

Professional Members

American Chemical Society

List of Collaborators in last 48 months.

Prof. Mark Young - Dept. Plant Pathology, Montana State University - Bozeman Montana.

Dr. Jeff. W.M. Bulte - Laboratory of Diagnostic Radiology Research, NIH, Bethesda Maryland.

Prof. Brad Tebo - Scripps Institution of Oeanography, La Jolla California.

Dr. Daniel Ripoll - Cornell University Theory Center, Ithaca New York.

Publications:

T. Douglas "Biomimetic Synthesis of Nanoscale Particles in Organized Protein Cages" in Biomimetic Approaches in Materials Science, S. Mann (ed.), VCH publishers, New York, 1996.

T. Douglas, S. Mann "Biomolecules in the Synthesis of Inorganic Solids" in Encyclopedia of Molecular Biology and Molecular Medicine, VCH publishers, New York, 1996.

S. Gider, D.D. Awschalom, T. Douglas, K. Wong, S. Mann, G. Cain, "Classical and Quantum Magnetism in Synthetic Ferritin Proteins" *J. Appl. Phys.* , 1996, 79, 5324-5326.

M. Zborowski, C. B. Fuh, R. Green, N. J. Baldwin, S. Reddy, T. Douglas, S. Mann, J.J. Chalmers, "Immunomagnetic Isolation of Magnetoferritin-labeled Cells in a Modified Ferrograph" *Cytometry*, 1996, 24, 251-259.

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T. Douglas and J. M. Didymus, "Mineral Sculptures in Biology", *Chemistry Review*, 1994, 4, 2-7.

R.J. Ward, M. Ramsey, D.P.E.Dickson, C. Hunt, T. Douglas, S. Mann, F. Aouad, T.J. Peters, R.R. Chrighton, "Further Characterization of forms of Hemosiderin in Iron-overloaded Tissues", *European Journal of Biochemistry*, 1994, 225, 187-194.

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S. Mann, D. D. Archibald, J. M. Didymus, T. Douglas, B. R. Heywood, F. C. Meldrum, N. J. Reeves, "Crystallization at Inorganic-Organic Interfaces: Biomaterials and Biomimetic Synthesis", *Science*, 1993, 261, 1286.

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manuscript submitted for publication

T. Douglas and D. Ripoll, "Calculations of the Electrostatic Field in the Iron Storage Protein Ferritin" submitted to Protein Science.

T. Douglas and M. Young, "Host Guest Encapsulation of Inorganic Materials by Assembled Virus Protein Cages" submitted to Nature.